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PATHWAYS AND REGULATION OF KETOGENESIS FROM BUTYRATE IN BOVINE LIVER AND RUMEN EPITHELIUM

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Pathways and Regulation of Ketogenesis from Butyrate in Bovine Liver and Rumen Epithelium" submitted by Roy Sidney Bush, B. S. A., in partial fulfilment of the requirements for the degree of Master of Science.

Abstract

It was found that propionate caused an inhibition of *in vitro* ketogenesis from butyrate by bovine liver slices, but not by bovine rumen papillae.

When succinate, fumarate and asparate were incubated with liver slices to provide intermediates of propionate metabolism, they were found to produce less antiketogenesis as they became further removed, metabolically, from propionate. The TCA cycle intermediates did not produce antiketogenesis when incubated with rumen papillae.

The possibility of competition between propionate and butyrate for the cofactors required for fatty acid activation in liver was examined. ATP (17 mM) did not affect the antiketogenesis due to propionate, thus, there did not appear to be a competition for ATP. Carnitine (3.5 mM), which was considered to expand the 'CoA pool', did not decrease antiketogenesis due to propionate. Therefore, it did not appear that there was competition between propionate and butyrate for the available CoA.

The hydroxymethylglutaryl-CoA pathway was the major route for deacylation of acetoacetyl-CoA in liver extracts. At a concentration of 0.20 mM, propionyl-CoA decreased the 3-hydroxy-3-methylglutaryl-CoA synthase activity by 50 %, whereas, propionate and methylmalonyl-CoA did not. At a concentration of 15 mM, propionate did produce 30 % inhibition of synthase activity. Propionyl-CoA did not affect the activity of 3-hydroxy-3-methylglutaryl-CoA lyase. This suggested that propionyl-CoA was a close enough analog of acetyl-CoA to act as a competitive inhibitor of the synthase reaction.

Synthase and acetoacetyl-CoA deacylase were both measurable in rumen epithelial extracts, but addition of succinate (15 mM) produced an

increased activity for acetoacetyl-CoA deacylation which was 3 fold, or more, of that of deacylase. The end product of the reaction was identified as succinyl-CoA indicating that 3-oxo acid CoA transferase was present in the papillae. The maximum activities for synthase, deacylase and transferase were 16 %, 14 % and 70 % of the total capacity to deacylate acetoacetyl-CoA, respectively. The absence of antiketogenesis was likely due to the lack of importance of the hydroxymethylglutaryl-CoA route of ketogenesis. The possible physiological significance of the participation of 3-oxo acid CoA transferase in ketogenesis in rumen epithelium was discussed.

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Table Of Contents

List Of Tables

List Of Appendix Tables

List Of Figures

Introduction

The ketone bodies probably occupy a role of significance in the normal metabolism of animals. Under circumstances of lipid mobilization, the oxidation of fatty acids only as far as ketone bodies in the liver followed by utilization of the ketone bodies by extrahepatic tissues provides, in essence, a means of respiration for the liver alternate to the TCA cycle, without a wastage of energy by the whole animal (Krebs, 1961). Ketosis, characterized by high blood levels of ketone bodies, is seen to develop under circumstances of metabolic carbohydrate depletion, such as, lactation, fasting, or diabetes. In contrast to monogastric animals, ruminants continually encounter a greater load of ketone bodies as their energy metabolism is based upon fatty acid oxidation. Normally the ketone body level in the blood of ruminants is substantially higher than in non-ruminants. Bergman, Kon and Katz (1963) reported that acetoacetate oxidation yielded 2 % of the expired CO_2 of fed ewes and that this was increased to as high as 30 % of the CO_2 in ketotic ewes. The study of ketone body formation and utilization is certainly of importance in understanding the normal and, in some instances, pathological, metabolic occurrences in ruminants.

The formation of ketone bodies is thought to occur when there is an accumulation of acetyl-CoA as a result of production at a rate in excess of the rate at which it may be utilized in the TCA cycle and for fatty acid synthesis. Normally, glucose, or gluconeogenic precursors, may provide oxaloacetate which is necessary for entry of acetyl-CoA into the TCA cycle. Even-numbered fatty acids yield acetyl-CoA via beta-oxidation, but cannot be used to form pyruvate or oxaloacetate, hence, their ketogenic activity is enhanced.

Propionate has been suggested to be antiketogenic by producing oxaloacetate and removing acetyl-CoA from ketogenesis. In early observations, propionate was found to inhibit ketogenesis from butyrate in ruminant liver, but not in rumen epithelium, in vitro (Krishnamurti and Milligan, 1968). Research was initiated to determine the reason for antiketogenesis due to propionate in bovine liver and the lack of this effect in rumen epithelium.

Literature Reveiw

A. Fatty Acid Metabolism In The Rumen Epithelium

I. Rumen Volatile Fatty Acids And Epithelial Absorption

Volatile fatty acids (VFA), including primarily acetate, propionate and butyrate, are quantitatively the major end products of microbial fermentation in the rumen. These are produced largely as a result of synthesis by rumen organisms of cellulase, an exoenzyme not found in animal digestive secretions, which catalyzes cleavage of the beta - 1,4 glycoside bonds in cellulose, releasing glucose (Kitts and Underkofler, 1954) for further microbial fermentation to the VFA.

Because the VFA are produced in the rumen by the organisms located there, this would be a logical site for absorption of VFA. Masson and Phillipson (1951) emptied rumens of fluid, removed the large solid material, added a known quantity of one of the neutralized VFA and measured the appearance of VFA in the blood draining the rumen. They found that there was a substantial absorption of each VFA into the portal blood as calculated by arterio-venous differences. In addition, they measured the disappearance of each VFA from the VFA supplemented rumen fluids and found a significant decrease, which was attributed to absorption.

Leng, Corbett and Brett (1968) designed continuous infusion experiments to measure the rate of dilution of labelled VFA in the rumen fluids, thus allowing a measurement of the rates of production of VFA in a steady state system. A series of equations were derived for prediction of the effective VFA production rates when the rumen concentration was known. Rearrangement of their equations yields:

$$\text{VFA concentration} = (A \times \text{VFA production rate}) + B$$

where the values for A are 24.2, 27.0 and 50.6 and B are 13.5, 2.1 and -2.66 for acetate, propionate and butyrate, respectively. The A coefficients for each VFA show that butyrate concentrations increase much more than acetate or propionate, when the same increase in effective production rate is substituted. This suggests that a resistance or barrier to butyrate absorption exists, relative to absorption of acetate or propionate. Annison, Hill and Lewis (1957) determined molecular percentages of the VFA, including the larger VFA, iso-butyrate, iso-valerate, valerate and butyrate, in the rumen fluids and carotid, jugular and portal blood. All VFA were found in the rumen, but of the larger VFA, only butyrate was found in the portal blood and none were found in carotid or jugular blood, suggesting that the rumen epithelium may present a barrier to absorption, based on molecular size.

II. Metabolism Of The VFA In The Rumen Epithelium

a. Oxidation Of Butyrate

Hird and Weidemann (1964) reported that when butyrate was presented to either the papillae or the muscle side of rumen epithelium, in vitro, there was an accumulation of ketone bodies (3-hydroxybutyrate and aceto-acetate) on the muscle side. They also found that long papillae (3 - 4 mm) increased the quantity of butyrate removed from the medium and the quantity of ketone bodies produced over those measurements with short papillae (.25 mm).

Cook, McGilliard and Richard (1968) demonstrated, in vitro, that twelve to eighteen carbon fatty acids were very ketogenic using rumen epithelium. Hird and Weidemann (1964) reported that ketogenesis from butyrate by rumen epithelium was almost abolished when a nitrogen atmosphere was used to produce anaerobic conditions.

Annison et al. (1957) fed sheep diets of hay, hay plus casein and straw plus casein and measured the VFA concentration in the rumen, portal blood, and carotid or jugular blood. Their results indicated very little absorption of butyrate, but they found a relationship between the rumen concentration of butyrate and the portal concentration of ketone bodies. Hird and Weidemann (1964) reported that 60 to 70 % of the butyrate taken up on the rumen side of epithelium appeared on the opposite side as ketone bodies. This suggests that butyrate from rumen fluid undergoes considerable metabolism in rumen epithelium before release into the portal blood.

b. Metabolism Of Acetate

Pennington and Pfander (1957) incubated acetate and butyrate with rumen epithelium and found that acetate was much less ketogenic than butyrate. Leng and West (1969) reported that 10 to 25 % of the 3-hydroxy-butyrate found in portal blood came from acetate produced in the rumen. They found that ketogenesis directly from acetate, accounted for utilization of less than 5 % of the total acetate produced in the rumen. They concluded that butyrate was more important for ketogenesis in rumen epithelium, than was acetate.

c. Metabolism Of Propionate

Pennington (1954) reported that in the absence of CO_2, propionate caused a decrease in the oxygen utilization by the epithelium, but when CO_2 was included in their incubation, respiration and propionate metabolism increased directly in proportion to the CO_2 concentration up to 20 volumes per cent of the atmosphere. Pennington and Sutherland (1956) found, using carboxyl-C^{14}-propionate, that the greater part of the label appeared as C^{14}-CO_2 and most of the remainder was found in the carboxyl group of

lactate. They incubated rumen epithelium with C^{14}-propionate, or C^{14}-CO_2, and found an accumulation of labelled succinate with specific activity similar to the precursors, when the TCA cycle was blocked with malonate. This information strongly supports the functionality in rumen epithelium of the metabolic pathway for propionate described by Flavin and Ochoa (1957) which entails fixation of CO_2 onto propionyl-CoA and isomerization of this carbon to the C-4 position of succinyl-CoA.

Appearance of most of the carboxyl-C^{14} from propionate as C^{14}-CO_2 indicates that the TCA cycle is operative in rumen epithelium. During propionate metabolism, the C-1 (carboxyl) is randomized between C-1 and C-4 of oxaloacetate due to the symmetrical structure of succinate and fumarate. Oxaloacetate condenses with an acetyl-CoA molecule in initiating the TCA cycle and the two CO_2 molecules released in reforming oxaloacetate arise from positions C-1 and C-4 of the first oxaloacetate. To produce C^{14}-lactate, the oxaloacetate would be decarboxylated to yield pyruvate plus CO_2 from the C-4 position of oxaloacetate. Pyruvate would be directly reduced to lactate without changing the labelling pattern. Pyruvate also has the possibility of metabolism to acetyl-CoA for condensation to citrate. The CO_2 released during the latter conversion would correspond to the C-1 position of oxaloacetate. The metabolic pathways described above were reported and discussed by Black et al. (1966) when they injected propionate-2-C^{14} intravenously into normal lactating cows and measured the C^{14} distribution in milk glutamate. The above conversions could account for the production of labelled CO_2 and lactate as products of propionate metabolism in rumen epithelium.

d. Interrelationship Between The Fatty Acids

Pennington and Pfander (1957) reported that propionate abolished

ketogenesis from acetate, but not from butyrate, using rumen epithelium. They found that there was no interconversion of acetate and butyrate unless propionate was included in the incubation medium. Smith, Goetsch and Jackson (1961) reported that propionate was antiketogenic when incubated with acetate plus butyrate using rumen epithelium. This information suggests that the antiketogenic effects of propionate may entail inhibition of conversion of acetate to ketone bodies and stimulation of oxidation of butyrate to acetate rather than ketone bodies.

B. Fatty Acid Metabolism In The Liver

I. General Considerations

The VFA absorbed from the rumen are transported in the portal blood to the liver. Annison et al. (1957) reported that there were measurable quantities of propionate and butyrate in portal blood, but none were found in jugular blood, suggesting removal by the liver. Pennington (1952) showed that ketogenesis from acetate and butyrate in liver was much greater than in kidney cortex and heart muscle in sheep, suggesting that liver was a second major site for ruminant ketogenesis. Much of the work done on liver ketogenesis has involved laboratory animals such as rats, mice, and guinea pigs. Interpretation of results obtained from non-ruminants may require a certain amount of care in extrapolating to ruminants.

II. Metabolic Pathways Of The VFA

a. Propionate Metabolism

The route for propionate metabolism in animal tissues was presented by Flavin and Ochoa (1957). In a review article, Kaziro and Ochoa (1964) described the metabolism of propionate via the TCA cycle. The reactions entailed were:

Propionate + ATP + CoASH ⟶ Propionyl-CoA + AMP + PPi (1)

Propionyl-CoA + ATP + CO_2 ⟶ Methylmalonyl-CoA + ADP + Pi (2)

Methylmalonyl-CoA ⟷ Succinyl-CoA (3)

The enzyme for propionyl-CoA carboxylation (eqn. 2) was isolated from sheep liver by Halenz et al. (1962). The isomerase enzyme (eqn. 3) was reported in sheep liver by Marston, Shirley and Smith (1961) and Mazumder and Sasakawa (1961). Succinyl-CoA may be metabolized to oxaloacetate in the TCA cycle (Leng and Annison, 1963; Kaziro and Ochoa, 1964). Oxaloacetate is converted to citrate upon condensation with acetyl-CoA, or it may be converted to pyruvate, lactate (Leng and Annison, 1963) or glucose (Krebs et al., 1964). Therefore propionate could give rise to net glucose synthesis.

Kaziro and Ochoa (1964) concluded that, although propionate is metabolized through succinyl-CoA, it may also be converted to lactate by way of a pathway (eqn. 4) entailing acrylate as an intermediate.

Propionyl-CoA ⟶ Acrylyl-CoA ⟶ Lactyl-CoA ⟶ Lactate (4)

Rendina and Coon (1957) noted that propionyl-CoA may be dehydrogenated to acrylyl-CoA in animal tissues. Vagelos et al. (1964) reported that extracts of pigeon heart muscle catalyzed the hydration of acrylyl-pantetheine to lactyl-pantetheine. In previous work, Mahler and Huennekens (1953), using rabbit liver and kidney enzyme preparations, reported that labelled propionate yielded lactate and pyruvate with labelling patterns that were consistent with an acrylate pathway.

Annison et al. (1957) reported that propionate was present in portal blood but absent from, or present only in traces in carotid and jugular blood in sheep. They concluded that propionate was removed by the liver.

Leng and Annison (1963) incubated 1-C^{14} and 2-C^{14}-propionate with sheep liver slices and found that the labelling pattern of glucose was consistent with gluconeogenesis involving randomization of the label in the TCA cycle. Annison et al. (1963) infused 1-C^{14} and 2-C^{14}- propionate into the portal vein and removed samples from the jugular after 60 min of infusion. They measured the labelling pattern of blood glucose and lactate and concluded that gluconeogenesis, in vivo, occurred by the TCA cycle, with no production of lactate from propionate via acrylate.

Leng, Steel and Luick (1967) suggested that some propionate is metabolized to lactate in the rumen epithelium and the total gluconeogenesis from propionate in ruminant liver must involve production from both blood propionate per se and lactate derived from propionate.

b. Acetate Metabolism

Acetate is a key metabolite in liver, because, as the CoA ester, it may either condense with oxaloacetate for oxidation in the TCA cycle or condense with other acetyl-CoA molecules for storage as long chain fatty acids.

Leng and Annison (1963) incubated 1-C^{14} and 2-C^{14}-acetate with sheep liver slices and found that the labelling patterns of glucose produced during incubation were consistent with acetate entry into the TCA cycle. Annison et al. (1963) infused 1-C^{14} and 2-C^{14}-acetate into the portal vein of sheep and measured the labelling patterns of glucose and lactate in jugular blood samples. They concluded that acetate carbon appeared in lactate and glucose via the TCA cycle, in vivo, even though 2-C^{14}-acetate produced higher than expected activity in the C-1 of lactate.

Ganguly (1960) reported that synthesis of long chain fatty acids occurs in bovine liver. He separated the fatty acid synthetase enzymes

into a malonyl-CoA producing (R_1) fraction and a fatty acid producing (R_2) fraction. The production of long chain fatty acids was measured by extraction of the products of the reaction into pentane (Wakil, Porter and Gibson, 1957). Ganguly (1960) concluded that the rate of fatty acid synthesis, which was measured by incorporation of label from acetyl-CoA or malonyl-CoA into long chain fatty acids, was limited by the carboxylation of acetyl-CoA to malonyl-CoA.

c. Butyrate Oxidation

Leng and Annison (1963) reported that the labelling patterns of glucose produced when 1-C^{14},2-C^{14} and 3-C^{14}-butyrate were incubated with sheep liver slices, suggested the oxidation of butyrate in the TCA cycle, *in vitro*. Annison *et al.* (1963) infused butyrate preparations labelled in the same positions as above, and found that the labelling patterns in blood glucose suggested the oxidation of butyrate by the TCA cycle, *in vivo*. Both *in vivo* and *in vitro* studies with 1-C^{14} and 3-C^{14}-butyrate produced the same labelling patterns in glucose as did 1-C^{14}-acetate, which suggests that *beta*-oxidation of fatty acids in sheep liver is operative.

Cook and Miller (1965) reported that butyrate was metabolized mainly by rumen epithelium of sheep, but the small amount which was absorbed into the portal blood was removed and utilized by the liver.

d. Ketogenesis

Pennington (1952) incubated liver slices from sheep and rats with the sodium salts of acetate or butyrate and found that 25 % of the acetate and 62 to 74 % of the butyrate was accounted for as ketone bodies. Annison *et al.* (1963) reported that butyrate was metabolized to ketone bodies in sheep liver as a four carbon unit. They also showed that butyrate

was metabolized to glucose via acetate and the TCA cycle, suggesting that during beta-oxidation of butyrate, some of the acetoacetyl-CoA was converted to ketone bodies. Williamson, Bates and Krebs (1968) reported that rat liver extracts contained thiolase which catalyzed the condensation of two acetyl-CoA molecules, producing acetoacetyl-CoA.

Measurement of total ketone bodies includes acetoacetate, its decarboxylation product, acetone and its reduction product, 3-hydroxybutyrate. Acetone is often disregarded in discussing ketone bodies because the concentration is very low. Formation of acetoacetate, which is the substrate for synthesis of 3-hydroxybutyrate, and hence, total ketone bodies, involves deacylation of acetoacetyl-CoA.

The major pathway for deacylation is considered to entail condensation of acetoacetyl-CoA and acetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and cleavage of this HMG-CoA releasing acetyl-CoA and acetoacetate. This pathway has been reported in rat liver (Stern and Miller, 1959; Williamson et al., 1968; McGarry and Foster, 1969), guinea pig liver (Sauer and Erfle, 1966) and bovine liver (Lynen et al., 1958). This general agreement on a pathway in all three animals, suggests that ketogenesis in liver is similar for ruminants and monogastrics.

The conversion of acetoacetyl-CoA to acetoacetate has also been suggested to be catalyzed by acetoacetyl-CoA deacylase. Stern and Miller (1959) reported that rat liver preparations actively produced acetoacetate from acetpacetyl-CoA in the presence of iodoacetamide, even though the HMG-CoA cleavage enzyme activity was almost entirely inhibited by the iodoacetamide. They interpreted this activity to mean that a direct deacylase was responsible. Drummond and Stern (1960) reported that iodoacetamide-treated ox liver homogenates had a considerable amount of deacylase

activity. Sauer and Erfle (1966) found that there was no deacylase activity in their guinea pig liver fractions, but explained the difference between their results and those of Drummond and Stern (1960) by the lower protein content and therefore lower enzyme concentration in their incubation mixtures. Williamson et al. (1968) measured low levels of deacylase in rat liver homogenates, but concluded that it played an insignificant role in ketogenesis. They found the Km (acetoacetyl-CoA concentration which yields one-half maximal reaction velocity) of deacylase for acetoacetyl-CoA to be 0.01 mM. They also calculated that the experimental concentration of acetoacetyl-CoA (0.05 mM) that they employed, was 2500 times greater than that existent in intact liver. The Km is 500 times greater than their calculated endogenous acetoacetyl-CoA concentration, which suggests that the reaction rate, in vivo, is very low and does not contribute significantly to ketogenesis.

III. Antiketogenesis

a. The Effect Of Propionate On Ketogenesis

Quastel and Wheatley (1933) demonstrated, using guinea pig liver homogenates, that all even numbered fatty acids were metabolized to ketone bodies but when propionate (odd numbered fatty acid) was added to the butyrate incubations, there were substantially less ketone bodies released. Leng and Annison (1963) reported that propionate caused a 50 to 75 % decrease in ketogenesis from acetate or butyrate using liver slices from well fed sheep. Bush, Milligan and Krishnamurti (1970) reported that propionate caused 85 % inhibition of ketogenesis from butyrate using bovine liver slices. Thus, the antiketogenic effect of propionate is not confined to ruminants.

Schultz (1958) supplemented 0.25 lb of sodium propionate per day in

the diet of one-half of a group of 100 lactating cows and measured blood glucose, blood ketone bodies and milk production for seven weeks. He found that propionate increased blood glucose and milk production and decreased blood ketones. The effects of propionate were considered to be a result of gluconeogenesis. Reid and Mills (1961) found that injection of sodium propionate, pH 7.4, into fasted pregnant ewes increased blood glucose and lowered blood ketones. They also injected propionate into fed, non-pregnant ewes and found increased blood glucose and slightly decreased blood ketones. They suggested that the antiketogenic effect of propionate was due to the increased blood glucose and the production of oxaloacetate, which allows for oxidation of acetyl-CoA and thus decreased ketogenesis.

b. The Effect Of TCA Intermediates On Ketogenesis

Reid and Mills (1961) found that succinate and malate caused a decrease in blood ketone bodies and a slight increase in glucose in fasted pregnant ewes. Beatty and West (1951) found that rats fed butyrate excreted high levels of ketone bodies in the urine; addition of TCA cycle intermediates to the diet caused a decrease in urinary ketone bodies. These authors suggested that part of the observed antiketogenesis was a result of glucose production and the rest was due to production of oxaloacetate which was considered to allow for diversion of acetyl-CoA from ketogenesis to oxidation in the TCA cycle.

Baird (1968) reported that the level of oxaloacetate in the livers of healthy cows was three times greater than that in the livers of ketotic cows. He concluded that a fall in the oxaloacetate concentration was associated with high rates of gluconeogenesis and was a major factor in initiating bovine ketosis. This conclusion suggests that the oxaloacetate

concentration is related to TCA cycle activity and during gluconeogenesis the condensation with acetyl-CoA to citrate is impaired due to a lack of oxaloacetate.

On the other hand, the evidence of the role of oxaloacetate in ketogenesis is not entirely clear cut. Kalnitsky and Tapley (1958) measured the oxaloacetate and acetoacetate levels in livers from normal and fasted rats. They found that 24 hr fasted rats had the same oxaloacetate concentration as normal rats, but the acetoacetate concentration was 10 times greater in fasted rats. They concluded that the level of oxaloacetate was normal during the early stages of ketosis, and suggested that some factor other than a low level of oxaloacetate was involved in initiating ketosis.

Shepherd, Yates and Garland (1965) concluded that conversion of acetyl-CoA to acetoacetate or to citrate was governed not only by the concentration of oxaloacetate, per se, but rather the route was dependent upon the energy status. Wieland, Weiss and Eger-Neufeldt (1964) also suggested that the supply of oxaloacetate, per se, was not the only factor responsible for ketogenesis. They incubated pig heart muscle citrate synthase (CS) with oxaloacetate, C^{14}-acetyl-CoA and palmityl-CoA, measured the C^{14}-citrate formed and calculated the Km. They concluded that palmityl-CoA lowered the affinity of CS for oxaloacetate, providing a sensitive regulatory system for the control of ketone body formation in liver.

Hird, Symons and Weidemann (1966) reported that when rat liver mitochondria were incubated with butyrate and a hexokinase system, the production of ketone bodies was decreased because the ATP competitive system decreased fatty acid activation and oxidation. They found that addition of TCA intermediates (malate, succinate, oxo-glutarate, citrate)

to the competitive system, there was increased oxygen consumption, ketogensis, and the proportion of ketone bodies reduced to 3-hydroxybutyrate. This information suggests that electrons derived from TCA oxidation could be used for increased reduction of acetoacetate production of ATP by oxidative phosphorylation for further butyrate activation.

c. The Effect Of Carnitine On Ketogenesis

Bressler and Katz (1965) reported that carnitine increased the amount of acetoacetate formed from C^{14}-pyruvate by guinea pig liver homogenates, but the specific activity was greatly reduced. However, they incubated mitochondria with carnitine and labelled pyruvate and found that the specific activity in the ketone bodies was the same as that of the added pyruvate. They concluded that carnitine assisted entry of endogenous long chain fatty acids into the mitochondria and that these stimulated production of acetoacetate. This was consistent with the findings of Williamson et al. (1968), who found that rat liver mitochondria contained the majority of the enzymes necessary for conversion of acetoacetyl-CoA to acetoacetate which suggested that if carnitine could increase the intramitochondrial fatty acids, ketogenesis would also increase.

Materials And Methods

A. Materials

I. Krebs-Ringer Bicarbonate Buffer

This preparation was taken from Umbreit, Burris and Stauffer (1959). and included sodium chloride, 0.90 % (w/v); potassium chloride, 1.15 % (w/v); calcium chloride, 1.22 % (w/v); magnesium sulphate, 3.82 % (w/v); and potassium dihydrogen phosphate, 2.11 % (w/v), which were mixed in the ratio, 100:4:3:1:1, and were diluted 1:4 with water. This solution was diluted 84:16 with sodium bicarbonate, 1.30 % (w/v) and gassed thoroughly with CO_2 to pH 7.0.

II. Incubation Substrates

Butyrate, propionate, succinate and fumarate stock solutions were prepared by dissolving 0.1 moles in 50 ml of H_2O, neutralizing to pH 7.0 with 5 M KOH and diluting to a total volume of 100 ml. These were stored at 0 C. Carnitine (0.21 M) was prepared immediately before use. The ATP (17 mM) was also prepared and neutralized immediately before use.

III. Coenzyme A Salts

Approximately 10 mg of CoA (12 umoles) were dissolved in 0.6 ml of H_2O and 0.1 ml of 1 M $KHCO_3$, pH 8.1. Filter paper treated with nitroprusside dissolved in methanol was used to spot test for free sulphhydryl groups. The acid anhydrides of the salts to be prepared were added to this solution dropwise and testing with nitroprusside was continued until there was a negative test. The solution was acidified and extracted 3 times with 3 volumes of ether. To remove the last traces of ether, the solution was gassed with nitrogen, the pH was adjusted to 5 and was stored at -15 C. This procedure is essentially that of Sly and Stadtman (1963). In the preparation of acetoacetyl-CoA, diketene was used in place of the

acid anhydride. During the preparation of HMG-CoA from HMG-anhydride, the other extraction was ommitted.

IV. 3-hydroxy-3-methylglutaryl Anhydride

Approximately 125 mg of 3-hydroxy-3-methylglutaric acid were dissolved in 1 ml of acetic acid. To this, 2.8 ml of anhydrous benzene and 0.4 ml of acetic anhydride were added and the mixture was stirred for 16 hr at 37 C. The solvents and reagents were removed completely by vacuum distillation. The anhydride was recrystallized twice from anhydrous benzene and the white, needle-like crystals were used for synthesis of the CoA salt. This preparation was taken from Louw, Bekersky and Mosbach (1969), which was a variation of the procedure of Hilz et al. (1958).

V. Methylmalonyl-Coenzyme A

One mmole of methylmalonic acid and 1 mmole of benzenethiol were dissolved in 5.0 ml of N,N'-dimethylformamide at 0 C. This mixture was stirred for 1 hr while 500 mg of dicyclohexylcarbodiimide, dissolved in 5 ml of N,N'-dimethylformamide, were added. Stirring was continued for 3 hr. After the addition of 10 ml of water, stirring was continued for 15 min. The precipitate was filtered with suction and washed with water. The filtrate was then acidified and extracted with several volumes of ether. The ether phase was washed with 0.1 N HCl and H_2O. Anhydrous sodium sulphate was used to dry the ether phase, which was then shaken with activated charcoal and filtered. To concentrate the thiophenyl-methylmalonate, the ether was removed under reduced pressure at room temperature. A solution of CoA (12 mM in 0.1 M $KHCO_3$, pH 8.1) was prepared and an excess of the thiophenyl-methylmalonate was added. To promote the reaction and remove the ether, the solution was gassed with nitogen for 3 hr at 0 C. This procedure of Trams and Brady (1960) was modified by

removing any unreacted CoA with a small quantity of acetic anhydride.

VI. Tissue

Tissue was obtained from the slaughter house immediately after killing and was stored in ice until used. Liver was cut into 3 inch square strips and slices were prepared by cutting with a sharp blade against a leucite block, with a 0.3 mm deep groove, which was pressed firmly on top of the tissue. Rumen epithelium was prepared by clipping the largest papillae from the inner surface. All prepared tissue was stored in iced bicarbonate buffer until required.

Liver homogenates were prepared by adding 2 volumes of 0.05 M Hepes buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5, to small cubes of tissue in a Potter Elvehjem homogenizing tube and mincing thoroughly at 0 C. Rumen epithelium was first frozen in liquid nitrogen and powdered using a mortar and pestle cooled with liquid nitrogen. The resulting powder was homogenized in a manner similar to that used for liver. Cell free extracts were prepared from the homogenates by centrifugation at 20,000 x g for 30 min. All homogenates were stored at -15 C.

B. Methods

I. Tissue Incubation

Each incubation flask contained 150 umoles of substrates, when used, in 0.15 ml of the appropriate stock solution and Krebs-Ringer bicarbonate buffer to a total volume of 3.0 ml. After blotting the tissue, approximately 200 mg of slices or papillae were weighed and added to the incubation mixture. Each flask was gassed to produce a 95 % oxygen: 5 % carbon dioxide atmosphere and incubated with shaking (100 cycles per min) for 2 hr at 37 C. After incubation, 0.1 ml of 70 % $HClO_4$ was added to precipitate the protein and after chilling in ice for 15 min, each

solution was centrifuged at 3,000 x g for 10 min. The supernatant was adjusted to pH 5 to 6 with 5 M KOH and the $KClO_4$ precipitate was centrifuged at 20,000 x g for 10 min. The supernatant was stored at 0 C until used for analysis. This procedure was similar to that used by Pennington (1952) except that 0.1 ml of $HClO_4$ was used rather than 0.5 ml.

II. Ketone Body Analysis

The following two procedures are those of Williamson et al. (1962) employing the buffer modifications of Mellanby and Williamson (1963) and Williamson and Mellanby (1963).

a. Acetoacetate

A cuvette containing 0.20 ml of unknown; 0.10 ml of phosphate buffer, pH 7.0 (0.10 M KH_2PO_4, 39 parts and 0.10 M K_2HPO_4, 61 parts); 0.02 ml NADH (4.2 mM) was allowed to equilibrate in the spectrophotometer[1]. The reaction was initiated with 5 ul of 3-hydroxybutyrate dehydrogenase[2] (EC 1.1.1.35) (1/10 dilution) and the decrease in optical density at 340 mu was followed to completion. The molar extinction coefficient was 6,220 cm^{-1}.

b. 3-hydroxybutyrate

A cuvette containing 0.15 ml of unknown; 0.05 ml Tris buffer, pH 8.5 (1.21 g hydroxymethylaminomethane, 14.3 ml of 0.2 N HCl and H_2O to 100 ml); 0.10 ml of hydrazine buffer (1 ml hydrazine hydrate, 5 ml 1 N HCl and H_2O to 20 ml); 0.02 ml NAD (15.1 mM) was allowed to equilibrate and react as before. The increase in optical density at 340 mu was followed to completion.

III. 3-hydroxy-3-methylglutaryl-CoA Synthase (EC 4.1.3.5)

This assay was similar to that proposed by Ferguson and Rudney (1959).

1 Gilford Recording Spectrophotometer (Model 2000)
2 From Boehringer Corporation, New York.

A cuvete containing 0.20 ml Tris buffer, pH 8.5 (0.05 M); 0.02 ml of $MgCl_2$ (0.005 M); 75 nmoles of acetoacetyl-CoA; 150 nmoles of propionyl-CoA (when included); 150 nmoles of acetyl-CoA; total volume 0.31 ml, was allowed equilibrate. The reaction was initiated with 5 ul of cell free tissue extract at the appropriate dilution and the optical density change at 310 mu was followed for 10 min to assess the reaction rate. The molar extinction coefficient was 11,800 cm^{-1} (Williamson et al., 1968). Acetyl-CoA was ommitted in the blanks for this assay.

IV. 3-hydroxy-3-methylglutaryl-CoA Lyase (EC 4.1.3.4)

0.30 ml of Tris buffer (0.083 M), pH 8.5; 0.03 ml of $MgCl_2$ (0.005 M); 1.5 umoles of HMG-CoA; 1.1 umoles of propionyl-CoA (when included) and H_2O to a total volume of 0.50 ml were mixed and the reaction initiated with 0.05 ml of the appropriate dilution of homogenate. The concentrations of the reagents were the same as those described by Williamson et al. (1968) but the volumes were reduced. At 4 min intervals, 0.13 ml aliquots were removed and deproteinized with 0.02 ml of 25 % (w/v) trichloroacetic acid. The protein was removed by centrifugation at 3,000 x g for 10 min.

The supernatant was analyzed for acetoacetate by the chemical method of Walker (1954). The diazo reagent was prepared immediately before use, by mixing 3.0 ml of 0.5 % (w/v) $NaNO_2$ and 20.0 ml of p-nitroanaline (0.05 %, w/v, p-nitroanaline dissolved in boiling water and 5 mmoles of HCl and diluted to 100 ml after cooling). This mixture was neutralized with 7.0 ml of 0.2 M sodium acetate. To measure the acetoacetate, 0.50 ml of 1 M sodium acetate, 0.05 ml of unknown solution and 0.45 ml of H_2O were added to 3.0 ml of diazo reagent and incubated at room temperature for 30 min. One ml of 5 N HCl was added to stop the reaction and the colored complex was extracted into 4.0 ml of ethyl acetate. The optical density of the

ethyl acetate was measured at 450 mu. The molar extinction coefficient was considered to be 7×10^6 cm^{-1} (Walker, 1954).

V. Acetoacetyl-CoA Deacylase

This analysis was set up and measured in the same manner as HMG-CoA synthase, except that the acetyl-CoA was replaced with 0.5 umoles of iodoacetamide (Williamson et al., 1968).

VI. Identification Of Succinyl-CoA

The reaction mixture was similar to that in the lyase assay but 0.10 ml of Tris buffer (0.083 M, pH 8.1) was used and 0.25 ml of rumen epithelial homogenate provided the source of enzyme. One-half of the reaction mixtures included 1.5 umoles acetoacetyl-CoA and the other half included 1.5 umoles of acetoacetyl-CoA and 5 umoles of potassium succinate. After 10 min of incubation, 0.1 ml of hydroxylamine solution (28 %, w/v, hydroxylamine solution mixed 1:1 with 3.5 N KOH) was added and allowed to react for 10 min. The reaction was stopped by addition of 25 ml of 95 % ethanol and precipitated protein was centrifuged out at 3,000 x g for 10 min. The supernatant was evaporated to approximately 4 ml under vacuum on a steam bath, following which this volume was further reduced, under vacuum, to 0.5 ml at room temperature. A portion of this solution (150 to 250 ul) was used to spot a paper chromatogram (Whatman, No 3MM). Development was accomplished by descending chromatography using water-saturated butanol as the mobile solvent. After drying the strips at room temperature, they were sprayed with a 1:4 dilution of ferric chloride developer (50 g of ferric chloride in 1 l of 0.1 N HCl in 95 % ethanol). For comparison, a succinyl-CoA reaction with no enzyme was used. The preparation of hydroxamates and chromatography thereof, was described by Stadtman and Barker (1955).

Treatment Of The Data

The tissue incubation experiments on liver slices showed a great deal of variability in the quantity of ketone bodies produced during repetitions of the same experiment. The data in the Appendix are expressed as the relative activity of liver to produce ketone bodies from butyrate in the presence of various additive substrates. The value obtained for ketone body production from butyrate in each experiment was set equal to 100 per cent and all other treatments were expressed relative to this.

Standard error was calculated from the formula:

$$S.\,E. = \left(\frac{(X - \bar{X})^2}{N(N-1)} \right)^{\frac{1}{2}}$$

To determine the reaction rate of the crude enzyme systems, the optical density readings for 4.6 min (12 values) were regressed against a linear time scale which contained 11 equal intervals over 4.6 min. This was changed slightly to accommodate the lyase system when there were only 3 readings during 2 time intervals of 4 min each.

The conversion of optical densities to concentrations were accomplished using the general formula:

$$\text{Concentration} = \frac{\text{Optical Density Change}}{\text{Extinction Coefficient}} \times \text{dilution factors}$$

The extinction coefficients for the reactions are reported in the methods.

The Rf values for the chromatography experiment were calculated by dividing the distances moved by the solvent front into the average distance moved by the spots in question.

Results

I. Fatty Acid Metabolism And Ketogenesis

The production of ketone bodies from propionate and butyrate by liver slices was measured (Table 1). Butyrate was found to be ketogenic, while propionate was not. Propionate reduced the production of ketone bodies from endogenous substrates by 80 %. Ketone body production in the presence of propionate and butyrate was only equal to endogenous production. The enhancement of ketogenesis by butyrate, in the presence of propionate, was 31 % of that when propionate was not added.

Ketogenesis from butyrate by rumen epithelium was approximately 30 times greater than that of liver (Table 2) Butyrate was found to be ketogenic in rumen epithelium and propionate was not. Propionate decreased endogenous production of ketone bodies by 60 %, but ketogenesis from butyrate was not affected by propionate.

The TCA cycle intermediates succinate and fumarate, or aspartate (to provide oxaloacetate) were substituted for propionate in some incubations to provide intermediates of propionate metabolism. Ketogenesis from butyrate, using rumen epithelium, was not inhibited by succinate or fumarate (Table 2). Ketogenesis from butyrate was inhibited 58 %, 41 % and 10 % by succinate, fumarate and aspartate respectively, when incubated with liver slices (Table 3). As before, propionate decreased the ketone bodies from butyrate by 84 %. The antiketogenic effect of TCA cycle intermediates decreased as these compounds became further removed, metabolically, from propionate.

The effect of ATP was measured by supplementing the liver incubations with ATP (Table 4). ATP decreased the production of ketone bodies from butyrate by 16 % and caused an increase in the proportion of acetoacetate.

Table 1

The Effect of Propionate on Ketogenesis from Butyrate[1] by Liver Slices

Treatment[2]	No. of incubations	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	8	8.99 ± 2.31	16.67 ± 4.91	25.65 ± 5.25
Butyrate	13	39.80 ± 5.51	60.19 ± 5.95	100.00 ± 3.63[3]
Propionate	4	1.39 ± 0.82	3.63 ± 1.84	4.93 ± 1.52
Butyrate + Propionate	12	11.44 ± 2.39	16.45 ± 3.48	27.89 ± 3.73

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 100 % = 31.73 ug / 2 hr / 50 mg liver dry weight

Table 2

The Effect of Propionate and TCA Intermediates on Ketogenesis[1] from Butyrate by Rumen Epithelium

Treatment[2]	No. of incubations	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	1	6.98	10.54	17.52
Butyrate	3	28.07 ± 16.50	71.93 ± 11.20	100.00 ± 18.00[3]
Propionate	3	0.00	6.80 ± 1.83	6.80 ± 1.83
Butyrate + Propionate	3	44.33 ± 2.44	56.21 ± 1.02	100.50 ± 1.49
Butyrate + Succinate	2	63.72 ± 9.92	54.82 ± 0.98	118.50 ± 10.90
Butyrate + Fumarate	2	58.23 ± 0.96	58.46 ± 1.34	116.70 ± 0.39

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 100 % = 1182 ug / 2 hr / 50 mg rumen epithelium dry weight

Table 3

The Effect of TCA Intermediates on Ketogenesis from Butyrate[1] by Liver Slices

Treatment[2]	No. of incubations	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	2	4.42 ± 2.09	13.67 ± 5.03	18.09 ± 2.94
Butyrate	4	30.18 ± 7.41	69.82 ± 11.20	100.00 ± 8.76 [3]
Butyrate + Propionate	3	6.96 ± 0.44	9.08 ± 3.85	16.04 ± 3.44
Butyrate + Succinate	4	9.84 ± 3.97	31.41 ± 4.15	41.37 ± 4.37
Butyrate + Fumarate	4	12.68 ± 5.12	46.29 ± 7.02	58.96 ± 5.77
Butyrate + Aspartate	2	30.69 ± 3.47	59.97 ± 4.42	90.66 ± 5.62

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 100 % = 50.85 / 2 hr / 200 mg liver wet weight

Table 4

The Effect of ATP on Antiketogenesis due to Propionate[1] by Liver Slices

Treatment[2]	No. of incubations	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	4	8.16 ± 1.61	10.50 ± 8.79	28.66 ± 10.40
Butyrate	8	38.59 ± 3.23	61.40 ± 4.69	100.00 ± 6.05 [3]
Butyrate + ATP	4	46.53 ± 12.40	38.14 ± 9.41	84.67 ± 20.60
Butyrate + Propionate	5	10.67 ± 3.38	19.22 ± 3.35	29.89 ± 5.70
Butyrate + Propionate + ATP	6	9.45 ± 1.32	13.22 ± 3.43	22.70 ± 4.48

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 100 % = 19.17 ug / 2 hr / 200 mg liver wet weight

Table 5

The Effect of Carnitine on Ketogenesis from Butyrate[1] by Liver Slices

Treatment[2]	Carnitine[3] Added	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	0	5.71	43.06	48.77
Butyrate	0	47.79	52.21	100.00[4]
Butyrate	10.5	63.61	51.13	114.70
Butyrate	7.5	59.37	100.30	159.60
Butyrate	4.5	72.23	125.40	197.60

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 umoles of carnitine added / 3 ml of incubation medium
4 100 % = 89.93 ug / 2 hr / 200 mg liver wet weight

ATP did not influence the inhibitory effect of propionate. The percentage of ketogenesis from butyrate, when propionate was added, was equal to that in the presence of ATP and propionate (Table 6).

Carnitine was added to the incubations to measure the effect on anti-ketogenesis due to propionate (Table 6). Carnitine was added to the incubations to yield a 3.5 mM solution (7 % of the butyrate concentration). Carnitine decreased the propionate inhibition of ketogenesis from butyrate only from 65 % to 62 %. In the presence of ATP, carnitine decreased antiketogenesis due to propionate from 65 % to 60 %. To test the effect of carnitine on ketogenesis from butyrate, carnitine was added at concentrations of 3.5 mM and 5 mM and was found to decrease ketogenesis by 15 % and 70 %, respectively. In further incubations, the carnitine concentrations were 3.5, 2.5 and 1.5 mM (Table 5) and these increased ketogenesis from butyrate by 15 %, 60 % and 98 %, respectively. It would appear the 3.5 mM carnitine had little effect on the production of ketone bodies from butyrate, whereas, lower concentrations increased ketogenesis and the higher concentration decreased it.

II. The Effect Of The Metabolites Of Propionate On The Enzymes Of Ketogenesis In Liver

The inhibition of ketogenesis from butyrate, using liver slices, by TCA cycle intermediates and propionate implicated the possible involvement of an intermediate closely related to propionate. An initial test of liver acetoacetyl-CoA deacylase showed very little activity, which led to the examination of HMG-CoA synthase activity and the effect of propionyl-CoA on this (Table 7). Acetoacetyl-CoA was utilized at the rate of 13.4 umoles / min/ g liver when acetyl-CoA was not included in the incubation. This was considered to represent endogenous activity. The net removal of acetoacetyl-CoA in the presence of acetyl-CoA was 11.62 umoles / min/ g

Table 6

The Effect of Carnitine on Antiketogenesis due to Propionate[1] by Liver Slices

Treatment[2]	No. of incubations	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	4	11.08 ± 4.34	14.10 ± 5.64	25.18 ± 4.41
Butyrate	5	46.78 ± 12.10	53.21 ± 12.10	100.00 ± 0.00
Butyrate + Carnitine	1 [4]	30.05	54.84	84.89
	1 [5]	18.91	10.00	29.16
Butyrate + Propionate	7	14.53 ± 3.66	20.60 ± 5.34	35.13 ± 4.49
Butyrate + Propionate + Carnitine	10	14.68 ± 1.96	23.65 ± 4.10	38.34 ± 3.12
Butyrate + Propionate + ATP	2	13.24 ± 0.06	21.70 ± 2.20	35.02 ± 2.14
Butyrate + Propionate + Carnitine + ATP	4	17.48 ± 2.39	22.46 ± 2.51	39.94 ± 1.16

1 Values are percentages of total ketone bodies from butyrate
2 For incubation procedure, see Methods Section I
3 100 % = 28.48 ug / 2 hr / 200 mg liver wet weight
4 Carnitine concentration was 3.5 mM
5 Carnitine concentration was 5 mM

Table 7

The Effect of Propionyl-CoA on HMG-CoA Synthase Activity[1]

Treatment[2]	Replicates 1	2	3	4	Mean	HMG-CoA Synthase Activity	% Activity
Control	7.18				7.18		
" + Enzyme	13.86	13.57	14.57	11.69	13.42 ± 0.62	0.00	0.00
" + AcCoA[3]	28.38	24.30	25.40	22.07	25.04 ± 1.31	11.62	100.00
" + PrCoA[4]	15.12	14.00	16.50	12.40	14.51 ± 0.87	1.09	9.38
" + Both	20.80	20.08	20.08	17.66	19.66 ± 0.69	6.24	53.70

1 Values are umoles of acetoacetyl-CoA disappearance / min / g liver wet weight

2 For incubation procedure, see Methods Section III
3 Acetyl-CoA
4 Propionyl-CoA

Table 8

The Effect of Methylmalonyl-CoA on HMG-CoA Synthase Activity[1]

Treatment[2]	Replicates 1	2	3	4	Mean	HMG-CoA Synthase Activity	% Activity
Control	1.31				1.31	0.00	0.00
" + MMCoA[3]	1.31				1.31	0.00	0.00
" + Enzyme	8.40	9.76 11.26	6.64 6.45	6.42 7.20	8.02 ± 0.71	6.71	100.00
" + Both	6.84	9.11 7.90	5.28 4.79	7.45 7.24	6.95 ± 0.56	5.64	84.00

1 Values are umoles of acetoacetyl-CoA disappearance / min / g liver wet weight

2 For incubation procedure, see Methods Section III
3 Methylmalonyl-CoA

liver and this was designated as 100 % synthase activity. Propionyl-CoA (0.48 mM) enhanced the endogenous activity by 10 % and inhibited the synthase reaction by 50 %.

The effect of methylmalonyl-CoA on synthase activity was measured (Table 8). The control assays contained acetyl-CoA and acetoacetyl-CoA. Methylmalonyl-CoA (0.4 mM)[1] did not show any effect on the reaction rate. Disappearance of acetoacetyl-CoA due to synthase activity was 6.7 umoles / min / g of liver. Synthase activity was decreased by 16 % when methylmalonyl-CoA was included.

The effect of potassium propionate on synthase activity was measured (Table 9). Synthase activity in experiment I was 4.38 umoles / min / g liver greater than that of the control which contained no enzyme. Propionate concentrations of 30 mM and 15 mM, which were 120 and 60 times greater than that of acetoacetyl-CoA, decreased synthase activity by 57 % and 30 %, respectively. The control in experiment II, which contained enzyme plus acetoacetyl-CoA, showed no increase in activity over the control in experiment I. This suggests that deacylase is not significant in liver ketogenesis. Synthase activity was 4.14 umoles / min / g liver greater than the control. Propionate concentrations of 1.5 mM and 0.15 mM had little effect on synthase activity.

To compare the inhibition of synthase activity due to propionyl-CoA to the total inhibition of ketogenesis from butyrate due to propionate, the effect of propionyl-CoA on HMG-CoA lyase was also examined (Table 10). Propionyl-CoA decreased lyase activity toward the end of the reaction period in replicate 1 and consequently there was a 3 % decrease in the mean lyase activity. The other replicates had the propionyl-CoA and

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1 The concentration is less than 0.48 mM because excess CoA was removed with acetic anhydride

Table 9

The Effect of Propionate on HMG-CoA Synthase Activity[1]

Treatment[2]	Replicates 1	2	3	4	Mean	HMG-CoA Synthase Activity	% Activity
Experiment I							
Control[3]	2.26	3.51	2.44		2.74 ± 0.39	0.00	0.0
Synthase	5.68	7.30 8.29	6.64 7.86	6.92	7.12 ± 0.38	4.38	100.0
" + Pr[4]	3.58	5.19	5.03		4.60 ± 0.51	1.86	42.5
" + Pr[5]	5.69	5.80	5.97		5.82 ± 0.08	3.08	70.3
Experiment II							
Control[6]	3.44	2.72	2.42	2.27	2.71 ± 0.26	0.00	0.0
Synthase	7.91	6.74	6.20	6.56	6.85 ± 0.37	4.14	100.0
" + Pr[7]	6.71	6.54	7.40		6.88 ± 0.26	4.17	100.7
" + Pr[8]	5.37	6.85	6.25		6.16 ± 0.43	3.45	83.3

1 Values are umoles of acetoacetyl-CoA disappearance / min / g liver wet weight

2 For incubation procedure, see Methods Section III
3 Control contains acetoacetyl-CoA and acetyl-CoA
4 Propionate (30 mM)
5 Propionate (15 mM)
6 Control contains acetoacetyl-CoA with enzyme
7 Propionate (1.5 mM)
8 Propionate (0.15 mM)

enzyme pre-incubated for 5 min and there was a 2 % increase in lyase activity. Propionyl-CoA produced no significant effect on HMG-CoA lyase.

III. The Mechanism Of Ketogenesis In Rumen Epithelium

The enzymatic removal of acetoacetyl-CoA was nearly equal in the presence and absence of acetyl-CoA, in preliminary assays, which suggested low HMG-CoA synthase activity in rumen epithelium. Subsequent investigations were directed toward measurement of deacylase activity in rumen epithelium (Table 11). That activity catalyzing removal of acetoacetyl-CoA that was inhibited by iodoacetamide was considered to be thiolase while that activity measured in the presence of iodoacetamide was considered to be deacylase (Williamson et al., 1968). Succinate (15 mM) increased the rate of removal of acetoacetyl-CoA by 0.19 umoles / min / g of rumen epithelium. Removal of acetoacetyl-CoA in the presence of succinate was 3.7 times greater than that due to deacylase. This additional activity was considered to be due to acetoacetyl-succinic thiophorase (Falcone and Boyer, 1959), or 3-oxo acid CoA transferase (EC 2.8.3.5) (Weidemann and Krebs, 1969b).

The existence of transferase was investigated by examination of the reaction products for the presence of succinyl-CoA (Table 12). The spot found when the hydroxamate of succinyl-CoA was chromatographed had an Rf value of 0.33. Acetoacetyl-CoA and the incubations containing acetoacetyl-CoA did not produce any spots. Acetyl-CoA produced a hydroxamate which yielded a chromatographic spot with Rf 0.62. The incubation containing acetoacetyl-CoA and succinate produced a hydroxamate spot with Rf 0.30, which corresponded to that from succinyl-hydroxamate. This was taken to indicate the presence of 3-oxo acid CoA transferase.

The relative activities of the enzymes of ketogenesis in rumen epithelium were measured (Table 13). The maximum possible activity of

Table 10

The Effect of Propionyl-CoA on HMG-CoA Lyase Activity[1]

Treatment[2]	Replicates 1	2[3]	3[3]	4[3]	Mean	% Activity
HMG-CoA	5.84	6.67	6.13	5.83	6.12 ± 0.10	100.0
HMG-CoA + PrCoA	4.77	5.66	7.45	5.92	5.95 ± 0.56	97.3

1 Values are umoles of acetoacetyl-CoA produced / min / g liver wet weight
2 For incubation procedure, see Methods Section IV
3 These incubations involved 5 min pre-incubation of the enzyme and PrCoA

Table 11

Rumen Epithelium Acetoacetyl-CoA Deacylase[1]

Replicate	Treatment[2] Control	Control + IOAc[3]	Control + Enzyme	Control + Both	Control, Enzyme + Succinate[4]
1	97.3		344.1		449.2
					480.5
2	120.2		318.9		535.9
					354.6
3	62.9	90.4	220.6	156.6	
4			233.2		392.5
			218.9		372.0
5	67.3	91.9	220.6	159.0	
6			228.0		437.3
7			308.4		587.2
8			173.4		376.1
Mean	86.9 ± 13.5	91.1 ± 0.7	215.8 ± 19.1	157.8 ± 1.2	442.8 ± 26.6
Deacylase Activity	0.0	4.2	164.9	70.9	355.9
% Activity	0.0	1.2	46.3	19.9	100.0

1 Values are nmoles of acetoacetyl-CoA disappearance / min / g rumen epithelium wet weight
2 For incubation procedure, see Methods Section V
3 Iodoacetamide
4 Succinate added was 15 umoles / ml of incubate

Table 12

The Chromatographic Patterns of Acyl Hydroxamates and Hydroxamates[1] of the Products of Rumen Epithelium Incubations

Treatment[2]	A[3]	B[3]
Acetoacetyl-CoA + Enzyme + Succinate	0.31	0.29
Acetoacetyl-CoA + Enzyme	no spots	no spots
Acetyl-CoA	0.62 [4]	
Acetoacetyl-CoA	no spots	no spots
Succinyl-CoA	0.34	0.32

1 Values are the calculated Rf
2 For incubation procedure, see Methods Section VI
3 Duplicate determinations
4 This Rf is from a separate experiment

Table 13

Activities of Enzymes of Acetoacetyl-CoA[1,2] Deacylation in Rumen Epithelium

Substrates	Additions	Activity	
Acetoacetyl-CoA	None[3]	0.14 ± 0.10	(9)[A]
	Iodoacetamide (1 umole)	0.05 ± 0.04	(4)[B]
	Succinate (3.5 umoles)	0.30 ± 0.00	(1)
	Succinate (7 umoles)	0.39 ± 0.00	(1)[C]
	Succinate (10.5 umoles)	0.34 ± 0.00	(1)
Acetoacetyl-CoA + Acetyl-CoA	None	0.20 ± 0.02	(11)[D]
	Iodoacetamide (1 umole)	0.15 ± 0.01	(4)
	Succinate (10.5 umoles)	0.38 ± 0.05	(3)

1 All values are umoles of acetoacetyl-CoA disappearance / min / g of rumen epithelium
2 The values in brackets are the replicates of each treatment
3 HMG-CoA synthase activity = D - A
4 Acetoacetyl-CoA deacylase activity = B
5 3-oxo acid CoA transferase activity = C - A

synthase was calculated from these data assuming that thiolase activity was not increased by the addition of acetyl-CoA. The activities found were 63, 51 and 252 nmoles of acetoacetyl-CoA disappearance / min / g of rumen epithelium for HMG-CoA synthase, acetoacetyl-CoA deacylase and 3-oxo acid CoA transferase, respectively. Thus, the transferase activity was substantially greater than that of the other two routes of acetoacetyl-CoA deacylation in rumen epithelial extracts.

Discussion

It has been suggested that there is increased ketone body production from acetyl-CoA, when oxaloacetate is diverted to gluconeogenesis and is thus unavailable for condensation with acetyl-CoA in citrate formation (Baird, 1968). Propionate and the TCA cycle intermediates, succinate and malate, were considered to be antiketogenic because they supplied carbon for gluconeogenesis and for net formation of oxaloacetate (Reid and Mills, 1961). In bovine liver, propionate resulted in 72 % inhibition of ketogenesis from butyrate (Table 1). Succinate and fumarate were found to be antiketogenic in bovine liver in this study (Table 3), but their effect did not appear to be entirely due to provision of oxaloacetate. The inhibition of ketogenesis from butyrate by TCA cycle intermediates did not rule out the effect of the supply of oxaloacetate, but succinate caused more inhibition than fumarate and propionate caused more inhibition than succinate. The concentration of these intermediates at the site of ketogenesis was unknown and the gradation of inhibition may have only reflected permeability barriers to the additives. On the other hand, the effects could be interpreted to suggest that there may be a direct inhibitory effect on ketogenesis by propionate, or an early metabolite of propionate.

Hird et al. (1966) reported that hexokinase reduced ketogenesis from butyrate by competing for the available ATP, and hence, decreased the activation of butyrate. Propionate activation involves ATP as an energy source (Kaziro and Ochoa, 1964), and thus, through competition for ATP may decrease ketogenesis from butyrate. The net inhibition of ketogenesis from butyrate in the presence of propionate was equal to that observed in the presence of propionate plus ATP (Table 4). ATP, in the presence and absence of propionate, decreased ketogenesis from butyrate and changed the

ratio of 3-hydroxybutyrate to acetoacetate somewhat, uggesting that there was uptake of ATP into the liver cells. Thus, propionate inhibition was not due to competition for ATP.

Propionate and butyrate both require CoA for activation, suggesting the possibility of competition between these two for the available CoA. Weidemann and Krebs (1969a) reported that L-carnitine was transacylated with propionyl-CoA to yield propionyl-carnitine and CoA by rat kidney cortex. They also found that propionyl-carnitine was readily metabolized. Thus, carnitine can provide a metabolizable complex and release CoA. Carnitine has been suggested to play a role in lipid transport which also involves transacylation of the acyl group from CoA to carnitine as represented by eqn. 5.

$$\text{Carnitine} + \text{fatty acyl-CoA} \rightleftharpoons \text{fatty acyl-carnitine} + \text{CoA} \qquad (5)$$

Thus, carnitine is visualized as in essence expanding the 'CoA pool', or at least acting as an acyl acceptor, freeing CoA. Weidemann and Krebs (1969a) reported that gluconeogenesis from propionate increased with increasing carnitine concentrations up to approximately 2 mM, after which it decreased. Ketogenesis from butyrate (Table 5) was increased by concentrations of carnitine of 1.5 and 2.5 mM, but was unaffected at 3.5 mM and decreased at a concentration of 5 mM, suggesting that there was carnitine uptake into the cell and that this was expanding the 'CoA pool' at concentrations less than 3.5 mM. Carnitine, at a concentration of 3.5 mM, did not increase ketogenesis from butyrate in the presence of propionate (Table 6), suggesting that the inhibition due to propionate does not involve a competition for the available CoA.

Cook, Liu and Quraishi (1969) isolated and purified acyl-CoA synthetases from ruminant tissues and found that the maximal activity in their sheep

liver preparation was for 3 to 7 carbon carboxylic acids. That is, there was about equal enzymatic capacity for propionate and butyrate activation.

Since inhibition of ketogenesis by propionate did not appear to be due entirely to the supply of oxaloacetate or competition for ATP or CoA, it was considered possible that there may be a direct inhibitory effect on the enzymes of ketogenesis. The major pathway for deacylation of acetoacetyl-CoA in bovine liver has been shown to involve the HMG-CoA route (Fig 1) described by Lynen et al. (1958) which, in a sense, is catalyzed by acetyl-CoA. Propionyl-CoA could be regarded as an analog of acetyl-CoA in that there is a difference of only one methyl group in the acyl moeity. In addition, both have a site subject to nucleophilic attack at the alpha carbon. Therefore, the effect of propionyl-CoA on the enzymes of the HMG-CoA pathway was investigated (Tables 7 and 10). Propionyl-CoA did inhibit the HMG-CoA condensation reaction. The most probable mechanism for inhibition of synthase activity would entail a competition between acetyl-CoA and propionyl-CoA to fill the active site on the condensing enzyme molecule.

Propionate also inhibited the synthase reaction (Table 9), but the concentrations of propionate required for this inhibition were more than 60 times that of acetyl-CoA. It appears likely that propionate was mimicking the effect previously observed with propionyl-CoA. The concentrations of acetate and propionate in portal blood of sheep (Annison et al., 1957) indicate that there would not be a large concentration of propionate relative to acetate in liver and that propionate would likely not be present in the liver at the concentrations used in this study. Pennington (1952) showed that the uptake of acetate and propionate, by sheep liver slices was almost equal. It is likely that in the in vivo circumstance, propionyl-CoA rather than propionate would inhibit ketogenesis in the liver

through inhibition of the HMG-CoA condensing enzyme.

Smith et al. (1961) reported that acetoacetate production from acetate plus butyrate was reduced by 8 umoles (73 %) in liver and 17 umoles (28 %) in rumen epithelium when propionate was included in the incubations. They concluded that propionate was more antiketogenic in rumen epithelium than in liver and in absolute terms this appears correct, however, on the basis of the percentage inhibition, the conclusion would be incorrect. In the present study, rumen epithelium was 30 times more ketogenic than liver, but, unlike liver, propionate did not inhibit ketogenesis from butyrate (Table 2). The lack of an antiketogenic effect of propionate in rumen epithelium, suggested that either there was an alternate pathway of ketogenesis in operation or the epithelial enzymes were insensitive to inhibition.

Hird and Symons (1961) measured the labelling patterns in ketone bodies produced from C^{14}-butyrate and concluded that they had obtained evidence for an operational HMG-CoA pathway in rumen epithelium from sheep. They also concluded that acetoacetyl-CoA deacylase may produce up to 26 % of the total ketone bodies. However, as the authors inferred, their data could be entirely explained if the HMG-CoA pathway were, in fact, inoperative, but there was exchange of carbons 1 and 2 of acetoacetyl-CoA with the acyl moiety of acetyl-CoA. Such exchange could, perhaps be catalyzed by aceto-acetyl-CoA thiolase (EC 2.3.1.9) and it was shown that thiolase was active in their preparations. Baird, Hibbitt and Lee (1970) reported the activities of thiolase, synthase, lyase and deacylase in bovine rumen epithelium and indicated that they had obtained evidence confirming the HMG-CoA as the major route of ketogenesis in rumen epithelium. In the present studies, disappearance of acetoacetyl-CoA (Tables 11 and 13), using rumen epithelium cell free extracts, in the presence of iodoacetamide was considered to be

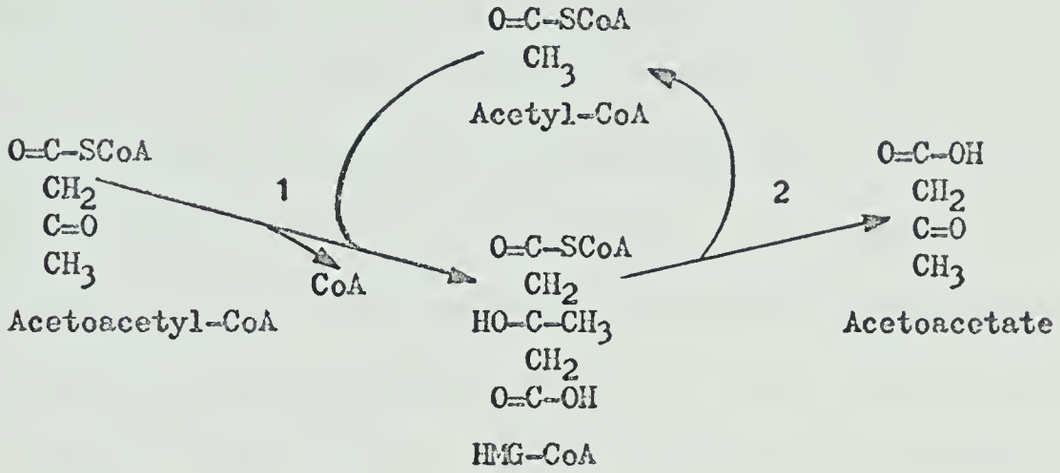
due to deacylase, because iodoacetamide blocks thiolase activity (Williamson et al., 1968). Deacylase activity was 50 to 70 nmoles of acetoacetyl-CoA disappearance / min / g of rumen epithelium. A liberal estimate of HMG-CoA synthase activity (Table 13) was taken to be the increased disappearance of acetoacetyl-CoA when acetyl-CoA was added to the assay mixture. Synthase activity was less than 63 nmoles of acetoacetyl-CoA disappearance / min / g of rumen epithelium. The activities of the enzymes extracted from rumen epithelium were less than those extracted from liver. The quantity of ketone bodies produced by epithelial papillae (Table 2) was much greater than that by liver (Table 1), which could not be explained by the measured enzyme activities. This would be explained if less of the enzyme present was extracted from rumen epithelium than liver, or there was another enzyme present in rumen epithelium which had not previously been measured.

It was of importance to note that the addition of succinate greatly stimulated acetoacetyl-CoA degradation by rumen epithelial extracts (Table 11). That this was due to the presence of acetoacetyl-succinic thiophorase, which catalyzes the reversible transfer of CoA from acetoacetyl-CoA to succinyl-CoA (Falcone and Boyer, 1959), was established by identification of succinyl-CoA as a product of the reaction (Table 12). The activity of transferase in rumen epithelial extracts for catalysis of acetoacetyl-CoA deacylation was some 270 % (Table 11) to 500 % (Table 13) of that of deacylase. It would therefore be quite likely that transferase participates to a significant extent in ketogenesis in rumen epithelium by the scheme presented in Fig 2 and recently discussed for rat kidney cortex by Weidemann and Krebs (1969b).

The pathway of ketogenesis in rumen epithelium involving transferase would have very substantial significance when the energetics are considered.

Fig 1

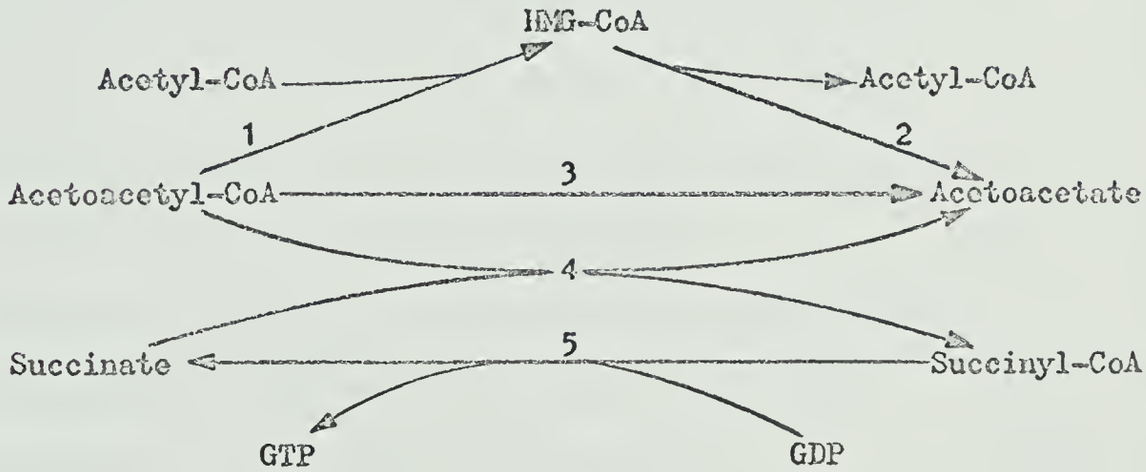
The Mechanism of Ketogenesis by Liver (HMG-CoA Pathway)



1 HMG-CoA synthase
2 HMG-CoA lyase

Fig 2

The Pathways for Deacylation of Acetoacetyl-CoA
by Rumen Epithelium



1 HMG-CoA synthase
2 HMG-CoA lyase
3 Acetoacetyl-CoA deacylase
4 3-oxo acid CoA transferase
5 Succinyl-CoA synthetase

The HMG-CoA and deacylase routes of conversion of acetoacetyl-CoA to acetoacetate would not allow for energy conservation. However, transferase (eqn 10), in combination with succinyl-CoA synthetase (eqn 11) would result in deacylation of acetoacetyl-CoA (Fig 2) with the conservation of one high energy phosphate bond per acetoacetyl-CoA. This, of course, assumes the presence of succinyl-CoA synthetase which would have to be the case if there is a functional TCA cycle in rumen epithelium as was suggested by Annison et al. (1963). The conversion of butyrate to acetoacetate and 3-hydroxybutyrate would normally be predicted to give rise to 3 and 0 high energy phosphate bonds (Fig 3). Studies of ketone body production from

Fig 3

Energy Production During Ketogenesis from Butyrate by Rumen Epithelium

Reaction	ATP yield[1]	
Butyrate + ATP + CoA ⟶ Butyryl-CoA + AMP + PPi	− 2	(6)
Butyryl-CoA + FAD + NAD ⟶ AcAc-CoA[2] + NADH + $FADH_2$	+ 5	(7)
AcAc-CoA + AcCoA ⟶ Acetoacetate + CoA + AcCoA	0	(8)
AcAc-CoA ⟶ Acetoacetate	0	(9)
AcAc-CoA + Succinate ⟶ Acetoacetate + Succinyl-CoA	0	(10)
Succinyl-CoA + GDP ⟶ Succinate + GTP + CoA	+ 1	(11)
Butyrate ⟶ Acetoacetate	+ 4 (3)[3]	(12)
Acetoacetate + NADH ⟶ 3-hydroxybutyrate + NAD	− 3	(13)
Butyrate ⟶ 3-hydroxybutyrate	+ 1 (0)[3]	(14)

1 moles of ATP produced / mole of butyrate metabolized assuming $FADH_2$ and NADH can yield 2 and 3 ATP from ADP in oxidative phosphorylation

2 Acetoacetyl-CoA

3 Figures in parentheses represent the net production of ATP if deacylation involves the HMG-CoA or deacylase routes

butyrate by rumen epithelium, including the present one, have routinely demonstrated substantial formation of 3-hydroxybutyrate, which would seem pointless if there was no energy yield. The transferase pathway would allow for net yields of 1 and 4 high energy phosphate bonds per molecule of butyrate converted to 3-hydroxybutyrate and acetoacetate, respectively.

In conclusion, ketogenesis from butyrate by bovine liver utilizes the HMG-CoA pathway, which allows for control of the condensation of aceto-acetyl-CoA and acetyl-CoA by an inhibition between propionyl-CoA and acetyl-CoA. The mechanism for deacylation of acetoacetyl-CoA in bovine rumen epithelium appears to involve very little HMG-CoA pathway activity as reflected by the inability of propionate to inhibit ketogenesis from butyrate incubated with papillae. The major pathway for deacylation of acetoacetyl-CoA likely involves transfer of the CoA to succinate to form succinyl-CoA which may yield one high energy phosphate bond in a substrate level phosphorylation when deacylated by succinyl-CoA synthetase.

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APPENDIX

Table 1

The Effect of Propionate and TCA Cycle Intermediates on Ketogenesis[1] from Butyrate by Rumen Epithelium

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	82.52	124.65	207.17
Butyrate	647.31	680.59	1354.90
	0.00	758.94	758.94
	321.65	1112.20	1433.85
Propionate	0.00	75.87	75.87
	0.00	45.47	45.47
	0.00	119.99	119.99
Butyrate + Propionate	467.64	688.72	1156.36
	542.36	651.42	1193.78
	562.64	654.05	1216.69
Butyrate + Succinate	636.26	636.66	1272.92
	870.81	659.91	1530.72
Butyrate + Fumarate	677.29	707.20	1384.49
	699.99	675.34	1375.33

1 Values are ug / 2 hr/ 50 mg of rumen epithelium, dry weight

Table 2

The Effect of Propionate and TCA Cycle Intermediates on Ketogenesis[1] from Butyrate by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	1.64	13.12	14.76
Butyrate	28.08	40.54	68.61
	5.85	65.84	71.69
Propionate	0.47	6.12	6.59
	0.00	1.72	1.72
Butyrate + Propionate	5.49	1.95	7.44
	4.66	5.93	10.59
Butyrate + Succinate	12.90	18.33	30.23
	0.00	27.55	27.55
Butyrate + Fumarate	0.00	46.37	46.37
	17.22	31.81	49.03
Butyrate + Aspartate	29.94	55.81	85.75
	13.12	28.34	41.46

1 Values are ug / 2 hr / 50 mg of liver dry weight

Table 3
The Effect of ATP on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	1.54	1.47	3.01
Butyrate	6.23	14.67	20.90
	11.65	20.33	31.98
Butyrate + ATP	8.67	5.47	14.14
Propionate + ATP	0.00	1.20	1.20
Butyrate, ATP + Propionate	0.89	3.64	4.53
	3.00	3.23	6.23

1 Values are ug / 2 hr / 50 mg of liver dry weight

Table 4

The Effect of Propionate and TCA Cycle Intermediates on Ketogenesis[1] from Butyrate by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	2.05	2.73	4.78
Butyrate	12.21	26.06	38.27
	10.61	14.20	24.81
Butyrate + Propionate	2.02	5.05	7.07
Butyrate + ATP	10.50	14.78	25.28
Butyrate, ATP	2.97	4.79	7.76
+ Propionate	2.36	2.74	5.10
Butyrate +	2.49	7.12	9.61
Succinate	4.26	11.88	16.14
Butyrate +	4.92	12.63	17.54
Fumarate	3.33	10.62	13.95

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 5

The Effect of ATP on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	0.89	3.05	3.95
Butyrate	2.81	3.34	6.15
	3.79	3.93	7.72
Butyrate + ATP	5.79	4.19	9.99
	2.53	1.70	4.23
Propionate	-0.09	0.23	0.15
	-0.28	0.00	-0.28
Butyrate, ATP + Propionate	0.36	0.83	1.20
	0.56	0.00	0.56

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 6

The Effect of Carnitine on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	12.38	1.0	13.38
Butyrate	48.93	2.61	51.54
Butyrate + Propionate	12.87	1.33	14.20
	12.38	1.33	13.71
Butyrate + Propionate + Carnitine	10.90	3.41	14.31
	10.90	3.71	14.61
	12.87	2.83	15.70

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 7

The Effect of Carnitine on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	1.86	5.90	7.76
Butyrate	8.43	16.51	24.94
Butyrate + Propionate	0.98 3.55	3.83 9.50	4.81 13.50
Butyrate + Propionate + Carnitine	2.23 3.22 3.47 3.26	6.91 9.94 6.40 9.02	9.14 13.16 9.87 12.28

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 8

The Effect of Carnitine on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Butyrate	5.16	7.43	12.60
Butyrate + Propionate	1.15	3.72	4.87
Butyrate + ATP + Propionate	1.66	3.01	4.68
Butyrate + Propionate + Carnitine	1.71	3.68	5.39
Butyrate + ATP + Propionate + Carnitine	1.77 1.68	3.33 3.42	5.10 5.10

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 9

The Effect of Carnitine on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	0.89	2.84	3.73
Butyrate	3.90	8.05	11.95
Butyrate + Propionate	2.76	2.93	5.69
Butyrate, ATP + Propionate	1.59	2.33	3.93
Butyrate + Propionate + Carnitine	1.58	4.28	5.86
Butyrate + ATP	2.27	2.17	4.44
+ Propionate	2.82	2.17	4.99
+ Carnitine			

1 Values are ug / 2 hr / 200 mg of liver wet weight

Table 10

The Effect of Carnitine on the Antiketogenesis due to Propionate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	2.25	2.92	5.16
Butyrate	13.08	28.31	41.39
Butyrate + Carnitine [2]	12.44	22.70	35.14
[3]	7.93	4.14	12.07
Butyrate + Propionate	0.96	13.04	14.00
Butyrate + Propionate + Carnitine	1.64	9.39	11.03

1 Values are ug / 2 hr / 200 mg of liver wet weight
2 3.5 mM carnitine
3 5 mM carnitine

Table 11

The Effect of Carnitine on Ketogenesis from Butyrate[1] by Liver

Substrate	Acetoacetate	3-hydroxybutyrate	Total Ketone Bodies
Control	5.14	38.73	43.87
Butyrate	42.98	46.95	89.93
Butyrate + Carnitine [2]	57.20	45.98	103.19
[3]	53.39	90.16	143.55
[4]	64.95	112.73	177.68

1 Values are ug / 2 hr / 200 mg of liver wet weight
2 3.5 mM carnitine
3 2.5 mM carnitine
4 1.5 mM carnitine